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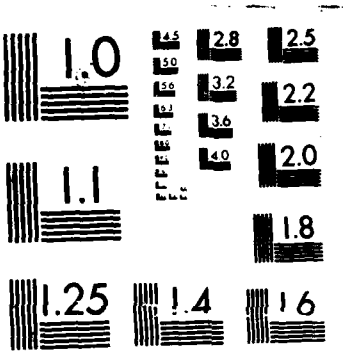

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AN INVESTIGATION OF THE CAUSES OF THE BLOOD PLATELET "STORAGE LESION"

ANNUAL AND FINAL REPORT

David T. Miller
Arthur P. Bode

April 22, 1986

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The role of thrombin in the platelet storage lesion has been examined and identified. We have shown that citrate formulations do not completely block the generation of thrombin during the preparation and storage of platelet concentrates. There is presented evidence that stored platelets and residual free calcium ions provide a support mechanism for formation of the prothrombinase complex in platelet concentrates. The addition of specific thrombin inhibitors and general inhibitors of platelet activation to citrate anticoagulants for platelet concentrates has resulted in remarkably improved storage characteristics as assessed by in vitro markers. We believe that these studies provide a sound basis for the further investigation of the effectiveness of novel anticoagulants for use in the preparation and storage of platelet concentrates.

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SUMMARY

We are pleased to report that the research supported by this contract has been successful in identifying a major contributing mechanism involved in the loss of function in stored platelet concentrates (PC). In this report, we present evidence that thrombin is present in citrated PC and that inhibition of thrombin and other activators of platelets greatly improves retention of platelet function and integrity during storage as judged by *in vitro* markers.

Evidence of thrombin activity in platelet concentrates comes from our studies of the appearance of fibrinopeptide A (FPA) in supernatant samples from stored PC. We have shown that FPA is elevated in citrated PC and that these measurements provide only an underestimate of the cumulative amount of thrombin present. Further findings indicate that a positive feedback system exists between platelet activation and thrombin generation in stored platelet concentrates. We have demonstrated the presence of platelet-associated prothrombinase activity (PF3) and non-negligible Ca^{2+} concentrations in stored PC, a combination that can potentiate thrombin generation in purified systems by 50,000 fold (J.P. Miletich *et al.*, Proc. Natl. Acad. Sci. 74: 4033-4036, 1977). The feedback is promoted by activation of platelets (by thrombin and/or other agents) which leads to expression of PF3 which potentiates the generation of more thrombin for further activation of platelets. The relevance of these findings to platelet storage is demonstrated by our success in extending the shelf-life of stored PC by the addition of specific inhibitors or thrombin (such as hirudin) and general inhibitors of platelet activation (such as the combination of PGE-1 and theophylline). Our results indicate that PC prepared with these inhibitors retain *in vitro* function and integrity for as long as 15 days at room temperature before the platelet storage lesion becomes apparent.

This work has been presented at national and international scientific conferences, and several manuscripts describing these findings have been prepared.

FOREWORD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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INTRODUCTION

The focus of this research effort was to investigate the role of thrombin as a major factor contributing to the loss of platelet function during storage of platelet concentrates (PC). Our long term goal continues to be better preservation of liquid-stored platelets through development of novel anticoagulants directed at limiting the potential for and effects of platelet activation in PC. Our working hypothesis was that the platelet storage lesion represents, at least in part, the consequences of platelet activation driven by the positive feedback loop of thrombin and expression of PF3, augmented by other agonists. The information given in this report is entirely consistent with this hypothesis.

At the time that this proposal was written, several investigators had data that were suggestive of the appearance of platelet activation in stored PC (1,2). Because of the documented inability of citrate formulations to completely block the generation of thrombin in whole blood or plasma (3,4,5), we thought it likely that significant levels of thrombin would be present in PC. The potency of thrombin as an activator of platelets is such that even traces can cause release of granule contents and other changes (6).

We set forth objectives in this contract to demonstrate the presence of thrombin activity in citrated PC and to investigate mechanisms supporting thrombin generation in the plasmatic environment of stored platelets. Further, we investigated the effects of supplementing citrate anticoagulants with potent thrombin inhibitors combined with reversible inhibitors of platelet activation. Evidence of thrombin activity was found, and there was a remarkable preservation of platelet function and integrity in PC stored with the inhibitors for 15 days.

The body of this report describes the results generated under each objective and includes a short discussion of the findings. Attached to this report are preprints of manuscripts giving complete experimental details and further information.

OBJECTIVES, RESULTS, and CONCLUSIONS

Objective (A). Demonstration of thrombin generation and PF3 expression by citrated platelet concentrates (PC).

(1). Detection of Fibrinopeptide A (FPA) formed in PC during preparation and storage.

Results: FPA is a 16 amino acid peptide that is cleaved specifically by thrombin from the A-alpha chain of fibrinogen as a first step in the formation of fibrin. The appearance of FPA in plasma can be taken as indirect evidence of thrombin activity in a sample. A study was made of FPA levels in citrated PC, which produced several important findings:

* FPA levels were well above controls in citrated PC and in the whole blood and platelet-rich plasma from which the PC were made (see Tables 1 & 2 of manuscript# 1).

* Exogenous FPA was rapidly degraded in PC (see Figure 1 of manuscript# 1), therefore explaining the lack of an accumulation of FPA over time of storage (viz., Table 1 of manuscript# 1).

* Addition of thrombin inhibitors (hirudin or PPACK) to the citrate anticoagulant reduced FPA levels significantly (see Table 2 of manuscript# 1), but there was no effect on the rate of degradation of exogenous FPA (Figure 1 of manuscript# 1).

* Addition of inhibitors of platelet activation and release (PGE-1 and theophylline) slowed the rate of degradation of FPA (Figure 1 of manuscript# 1), thus allowing an accumulation of FPA to occur during long term storage of PC (see Table 3 of manuscript# 1).

(2). Demonstration of the consumption of antithrombin activity in plasma of stored PC in conjunction with the formation of high molecular weight complexes of antithrombin III (AT-III).

Results: AT-III is a naturally occurring inhibitor of serine proteases which forms complexes with thrombin when present. We were able to detect a loss of AT-III activity in citrated PC over 5 days of storage (see attached table). The magnitude of the decrease was correlated with platelet count in the PC. However, trial experiments to identify AT-III complexes in PC ran into technical difficulties. The amount of decrease in AT-III activity noted above was so small (mean <10%) that only very sensitive methods could be used for detection of complexes. We tried single dimension electropheresis of PC samples followed by a Western blot with an anti-thrombin antibody capable of recognizing thrombin in complexes with AT-III. The data were suggestive of positive results, but the background staining was too high to be conclusive.

(3). Demonstration of the conversion of prothrombin to thrombin and plasma Factor X to Xa in PC by means of radiolabelled zymogens.

Results: Four attempts were made to introduce small amounts of highly radioactive prothrombin (obtained from colleagues at our institution) into PC and follow the evolution of radioactive material that could be identified as thrombin by LC or PAGE. However, in all experiments to date, fibrin clots appeared in the PC within 24 hours after injection of the prothrombin preparations. Analysis of the preparations showed the presence

TABLE FOR OBJECTIVE A(2)

CORRELATION OF PLATELET COUNT AND DECREASE IN LEVELS OF ANTITHROMBIN III ACTIVITY (% OF NORMAL POOLED PLASMA) IN STORED PC.

PC#	Platelet Count (x 10^9/mL)	AT-III 1 h	AT-III 120 h
1	1.35	89	86
2	1.40	105	102
3	1.43	105	97
4	1.46	97	94
5	1.65	96	97
6	1.65	110	94
7	1.69	69	60
8	2.48	104	85

PC were prepared in CPDA-1 from 8 donors (4 male, 4 female). The decrease in AT-III was significant; paired t-test, one-tailed, p=0.0094. The magnitude of the decrease in AT-III was correlated with platelet count in PC; Spearman r=0.59, p=0.02.

Heparin Cofactor II activity levels did not change during storage (p=0.40)

of significant impurities which could not be removed by chromatography. Pretreament of the prothrombin with an AT-III agarose gel to remove contaminating thrombin or Factor Xa did not prevent clot formation in PC. It is unfortunate that thrombin-free prothrombin was not available, since this experimental design could have provided irrefutable evidence of thrombin generation in PC.

(4). Demonstration of the appearance of platelet procoagulant activity (PF3) known to support thrombin generation.

Results: As stated in the INTRODUCTION, activated platelets can accelerate the rate of thrombin generation in plasma by several orders of magnitude. We found high and ever-increasing levels of PF3 in citrated PC (see Table 2 of manuscript# 2). Because of the observed coincidental, but not necessarily proportional, release of LDH in PC (see Table 3 of manuscript# 2), we were unable to differentiate between lysis and platelet activation as the means of expression of PF3. In either case, the conclusion remains that stored platelets express an activity (PF3) that greatly amplifies thrombin generation in plasma.

(5). NEW STUDY. In order to further test the hypothesis that prothrombinase complexes are assembled during storage of platelets, we examined the levels of Ca^{2+} in citrated PC. Ca^{2+} is an integral part of prothrombinase, involved in the binding of Factor Xa, Va, and prothrombin to the activated platelet surface.

Results: We found concentrations of Ca^{2+} sufficient to support prothrombinase assembly in citrated PC. Also, the data show an increase in total and ionized calcium during preparation and storage of PC (see Table 1 of manuscript# 3). The source of the released calcium was most likely cellular, since platelet-poor plasma showed no such increase (see Table 3 of manuscript# 3).

Conclusions.

The FPA data show quite clearly that thrombin activity is present in the preparation and storage of citrated PC. The degradation of exogenous FPA in PC indicates that measurements of FPA in plasma samples represent only an underestimate of cumulative thrombin activity. Our findings of reduced AT-III activity in stored PC also support the conclusion that thrombin is formed in PC, although we were unable technically to demonstrate its physical presence. Demonstration of PF3 activity and detectable Ca^{2+} in samples of citrated PC further support our hypothesis that assembly of the prothrombinase complex is possible in PC, therein making generation of significant levels of thrombin quite likely.

We submit that Objective (A) has been completed and that our conclusion that thrombin is generated during the preparation and storage of PC is sound.

Objective (B). Investigation of the relationship between thrombin generation and PF3 expression by modification of thrombin generation in stored PC.

(1). Effects of specific thrombin inhibitors (PPACK, DABE, hirudin) as primary anticoagulants on the relationships among thrombin generation markers, PF3 levels, platelet morphology, PF4 release, and LDH release.

Results: Several investigators have postulated that chelation of Ca^{2+} in blood by citrate induces changes in platelets that make them highly reactive (3). It was our original intention to anticoagulate whole blood using thrombin inhibitors alone, without citrate. We tried several formulations (DABE was unavailable during the entire contract period, so we substituted 5'AmidinoIndole):

* Hirudin at up to 20 units/mL (in plasma),
* PPACK (Phe-Pro-Arg-chloromethyl ketone) at 20 μM,
* Hirudin + 5'AmidinoIndole (a Factor Xa inhibitor) at 10^{-4} M, and
* 5'AmidinoIndole alone.

In all cases, the PC clotted at 24-48 hours after preparation, even though the initial morphology scores were excellent. In our opinion, these inhibitors failed as primary anticoagulants because of the highly procoagulant nature of PC and possibly as a result of instability of these compounds in plasma. It is interesting that the platelet-poor plasma produced in these preparations did NOT clot during storage. It became apparent from these studies that citrate was a necessary component of the anticoagulant.

(2). Effects on the above markers of adding excess antithrombin III to citrated PC.

Results: This approach was deemed unnecessary in light of the minimal decreases in AT-III activity observed in stored PC (see A(2) above).

(3). Investigation of the above markers in PC prepared from donors with severe clotting factor defiencies (Factor V, VII, VIII, or X-deficient donors).

Results: The initial experiment of storage of PC from a Factor VIII-deficient donor (see original proposal) was repeated with a Factor X-deficient donor. After 7 days in a PL-146 storage bag, the pH was 7.26, the PF3 values were low relative to normal controls, and the pO_2 was 58 (indicating that respiration was still maintained). However, clumps of cells had formed and by electron microscopy less than 10% of the platelets were in a discoid shape. The mixed results could be interpreted as showing the effects of influences other than thrombin in storage of PC. These experiments were discontinued in favor of the use of inhibitors in normal donors to avoid undue exposure of lab workers to hemophiliac blood and components with the implied risk of contamination by infectious agents such as HTLV-III.

(4). Investigation of addition of low amounts of purified thrombin to PC and the consequent effects on the above markers.

Results: Injection of as little as 1.0 NIH unit of thrombin into a 50 mL citrated PC resulted in an immediate increase in % platelets with pseudopodia without causing discernible clot formation in the bag. However, further changes in storage parameters were confounded by the effects of the buffer vehicle containing stabilizers of thrombin. The experimental design used was that two PC of similar ABO type from the Red Cross were pooled and redistributed into separate bags prior to injection with either thrombin or buffer. Both types of PC showed a more rapid deterioration of pH and morphology than was seen in untreated PC. Little difference was seen in PF3 between treated and untreated PC, probably because the levels of activity were quite high prior to injection.

(5). Demonstration of the association of thrombin formed from radiolabelled prothrombin (see A(3) above) with the plasma membrane of stored platelets, thereby indicating a cause-and-effect relationship between thrombin generation and platelet activation in stored PC.

Results: This approach was abandoned due to the unfortunate difficulties we encountered in obtaining a preparation of radiolabelled prothrombin that was not highly thrombogenic (see A(3) above).

Conclusions.

The results from preparation of PC using thrombin inhibitors as primary anticoagulants were very revealing. The profound procoagulant nature of stored PC caused frank clotting to occur in 1-2 days after PC preparation, while the platelet-poor plasma produced in these preparations never showed signs of clotting. We had attempted to avoid the use of citrate in our anticoagulant formulations in order to keep the platelets in a Ca^{2+} - rich environment to study the difference this would make in storage. Significant success was acheived by combining these inhibitors with citrate and inhibitors of platelet activation (see below). Although direct demonstration of thrombin generation and its interaction with platelets in stored PC was not technically possible, the beneficial effect of thrombin inhibitors in storage of PC is made abundantly clear.

We submit that the thrust of Objective (B) has been proven in spite of the technical difficulties limiting some approaches.

Objective (C). Investigation of the relationship between markers of platelet activation and markers of platelet damage in citrated PC modified by the addition of inhibitors of platelet activation.

(1). Demonstration of the release of PF3 and platelet microvesicles into the PC supernatant plasma such as occurs with in vitro stimulation of platelets.

Results: We found that when samples from citrated PC were centrifuged to pellet the platelets, 56% ± 15 of the PF3 activity remained in the

supernatant. Chromatography of the supernatant plasma from outdating PC isolated a turbid fraction of high molecular weight with high specific activity for PF3 (see Figure 2 of manuscript# 2). While these findings are entirely consistent with observations in washed human platelets after stimulation with collagen (7), they are also similar to the results obtained from chromatography of frozen-thawed platelets (8). Concurrent measurements of markers of platelet lysis (see Table 3 of manuscript# 2) suggested that both platelet activation and platelet lysis are occurring during PC preparation and storage.

(2). Demonstration of the release of PF4 and its relationship to PF3 levels.

Results: As stated above, we have found, as have others (2), released LDH in PC samples. It is difficult to interpret findings of released PF4 (see 2) as a marker of platelet activation when there is also evidence of platelet lysis. In addition, we have learned recently that the release of PF4 does not necessarily coincide with the appearance of PF3 in the supernatant from activated platelets (9). In light of this information, we regard further studies of PF4 in stored PC as uninformative.

(3). Relationship of the above [PF3 markers] to platelet morphology changes, release of LDH, and pH measurements in PC.

Results: We have identified a trend in citrated PC of PF3 levels increasing with time over 5 days of storage. Coincidental findings included worsening morphology score, decreasing pH, and increasing release of LDH (see Tables 2 & 3 of manuscript# 2). The magnitude of the changes in PF3 levels exceeded those of the other markers, thus weakening statistical correlations. In particular, we found inconclusive a direct comparison of PF3 and released LDH, thus making it impossible to distinguish between platelet activation or lysis as the source of expression of PF3 in stored PC.

(4). Modification of all the above by addition of specific platelet activation inhibitors (prostaglandin E1, theophylline, adenosine cocktail) to the anticoagulant solution in PC.

Results: We have completed a series of experiments of extended storage of PC in anticoagulant formulations containing CPDA-1 plus prostaglandin E1 and theophylline (we have found adenosine to be unnecessary) with and without thrombin inhibitors (hirudin or PPACK). The results have been quite encouraging, especially with the combination of a thrombin inhibitor and platelet activation inhibitors. At the end of 15 days of storage at 22 C, the experimental PC showed acceptable pH, morphology, hypotonic shock response and low LDH release while the controls stored in CPDA-1 alone were completely dysfunctional (see Table 1 of manuscript# 4). The preservation of platelet morphology in the PPACK anticoagulant was excellent over the entire extended storage interval, but this finding must be interpreted in light of recent information describing the direct effects of chloromethyl ketone peptides on cell cytoskeletons (10). However, the hirudin anticoagulant also showed good preservation of platelet morphology.

Final Conclusions and Recommendations.

Objective (C) represents the application to practice of our theory of a feedback loop between platelet activation and thrombin generation in citrated PC. We have succeeded in extending the shelf-life of stored PC, as judged by in vitro markers, to 15 days in anticoagulants containing citrate supplemented with reversible platelet activation inhibitors and a thrombin inhibitor. It would appear from our data that blocking either platelet activation or thrombin generation alone is not as beneficial as the combination of these interventions. The effects of these novel anticoagulants on platelet storage are much more striking than the changes seen thus far with the advent of new plastic containers or new modes of agitation. We believe that there is still much to learn from the use of novel anticoagulants and much more to gain from testing new inhibitors of thrombin or other additives.

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